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ДЕЯКІ ВІРУСНІ ХВОРОБИ *LYCOPERSICON ESCULENTUM*, ЩО ЦИРКУЛЮЮТЬ НА ТЕРИТОРІЇ УКРАЇНИ

Робота присвячена детекції вірусів рослин, що інфікують L. esculentum на території України. За результатами ІФА визначено наявність антигенів вірусів родів Tobamovirus (PMMoV, ToMV), Cucumovirus (CMV) та Tobravirus (TRV). При дослідженні вірусних хвороб L. esculentum здебільшого зустрічалась моноінфекція. Найбільш розповсюдженим згідно наших дослідженнях виявився вірус мозаїки томату.

Ключові слова: вірусні захворювання, Tobamovirus (PMMoV, ToMV), Cucumovirus (CMV) та Tobravirus (TRV), помідори.

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НЕКОТОРЫЕ ВИРУСНЫЕ БОЛЕЗНИ *LYCOPERSICON ESCULENTUM*, ЦИРКУЛИРУЮЩИЕ НА ТЕРРИТОРИИ УКРАИНЫ

Работа посвящена детекции вирусов растений, которые инфицируют L. esculentum на территории Украины. По результатам ИФА определено наличие антигенов вирусов родов Tobamovirus (PMMoV, ToMV), Cucumovirus (CMV) та Tobravirus (TRV) При исследовании вирусных болезний L. esculentum в основном превалировала моноинфекция. Согласно нашим исследованиям более распротраненным оказался вирус мозаики томата.

Ключевые слова: вирусные заболевания, Tobamovirus (PMMoV, ToMV), Cucumovirus (CMV) та Tobravirus (TRV), томаты.

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FUSION EXPRESSION OF RECOMBINANT HUMAN BETA-DEFENSIN-3 AND ANALYSIS OF ITS BIOLOGICAL ACTIVITY

Human beta-defensins (hBDs) are small cationic antimicrobial peptides with multiple biologic activities. The aim of the study was cloning, expression in E.coli, purification and in vitro analysis of biological activity of recombinant human beta-defensin-3 (rec-hBD-3). hBD-3 cDNA was cloned into pGEX-2T vector, and recombinant plasmid was transformed into E.coli BL21(DE3) cells. Rec-hBD-3 was expressed in bacterial cells as GST-hBD-3 fusion protein, and purified by 3-step procedure via affine chromatography on glutathione-agarose, cleavage of fusion protein by thrombin, and reverse phase chromatography on Sep-Pack C18. Analysis of biological activity of rec-hBD-3 has shown that the peptide is active against Pseudomonas aeruginosa in micromolar concentrations in radial diffusion test. Rec-hBD-3 did not affect proliferation and viability of cultured human cancer cells of A431, A549, and TPC-1 lines, but was capable to potentiate cytotoxic effects of rec-hBD-2 and docetaxel in vitro.

Key Words: human beta-defensin-3, cancer cell, proliferation, viability, antimicrobial activity.

Intriduction. Human beta-defensins (hBDs) are small cationic antimicrobial peptides produced by different cell types. Human beta-defensins have been primarily recognized to possess a broad spectrum of antimicrobial activities, but as it has been shown later exhibit multiple biologic effects toward eukaryotic cells [1, 2]. In a number of studies there have been shown effects of hBDs on many important cell processes – cell proliferation, viability, differentiation, and apoptosis, and it has been shown that such effects of hBDs are concentration-dependent and could be exerted against many cell types [3-6]. Human β-defensin-3 (hBD-3; *DEFB103*) was firstly isolated in 2001 from human psoriatic lesions and cloned from keratinocytes and tracheal epithelial cells [7]. Mature hBD-3 molecule is composed from 45 aminoacid residues (molecular weight is 5.15 kDa) and possesses high cationic charge (+11). This defensin possesses potent broad spectrum activity against Gram-negative and Gram-positive bacteria but, unlike other studied members of beta-defensin family, antimicrobial activity of hBD-3 is found to be salt-insensitive. hBD-3 is an antimicrobial with an inducible expression which could be up-regulated upon stimulation with interferon-γ via STAT binding site in promoter region of *DEFB103* gene [8]. Expression of hBD-3 has been registered in many tumor types but its functional role remains unclear: hBD-3 is thought to play a role of pro-oncogenic molecule in some tumors (head and neck cancer, oral carcinoma [9, 10]) or tumor suppressor in salivary gland tumors [11]. Up to date, the role of hBD-3 in tumor cell biology is insufficiently investigated.

The present study was aimed on prokaryotic expression of hBD-3 (rec-hBD-3) and analysis of its biological activity *in vitro*.

Materials and methods. Human epidermoid carcinoma A431 cells and human lung adenocarcinoma A549 cells were obtained from the Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). Human papillary thyroid carcinoma cell line TPC-1 was kindly provided by Dr. V.M. Pushkarev (V.P. Komissarenko Institute of Endocrinology and Metabolism, AMS of Ukraine, Kyiv, Ukraine). The cells were cultured *in vitro* in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate in 5% CO_2 atmosphere at 37 °C. To clone the gene coding mature hBD-3 sequence, total RNA was isolated from human lung adenocarcinoma sample, and hBD-3 cDNA was obtained by reverse transcription using a pair of specific primers: hBD-3-F: GCG CGG ATC CGG AAT CAT AAA CAC ATT ACA GAA; hBD-3-R: CAC GAA TTC TCA TTT CTT TCT TCG GCA GCA TTT TCG GC. Primer design was based on analysis of NM_001081551.3 sequence from GenBank database. hBD-3 cDNA was cloned into pGEX-2T vector (GE Healthcare, Sweden). Restriction of plasmids, ligation of fragments, bacterial cell transformation were performed by standard protocols. Rec-hBD-3 was purified by three-step procedure successfully used earlier for purification of rec-hB-2 and rec-hBD-4 [12, 13]. In brief, E. coli BL21(DE3) bacteria transformed with GST-hBD3-recombinant plasmid were induced with 1 mM IPTG for 6 h and precipitated by centrifugation. The bacterial cell lysate was applied to affine chromatography on glutathione agarose column (GE Healthcare, Sweden) with following cleavage of the defensin from fusion protein by thrombin digestion. hBD-3 peptide was further purified by reverse phase chromatography on Sep-Pack C18 cartridge (Waters, USA). Protein purity of the peptide preparation was analyzed by 7–22% gradient SDS PAGE. Antimicrobial activity of rec-hBD-3 was analyzed against *Pseudomonas aeruginosa* by bacterial growth suppression in radial diffusion assay [14]. *P.aeruginosa* ATCC 9027 strain was obtained from Ukrainian Collection of Microorganisms (D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine, Kyiv, Ukraine).

To study the effect of rec-hBD-3 on cell proliferation A431, A549, and TPC-1 cells were cultured in 24-well plates ($5x10^4$ cells per well) to nearly 50% confluence and then treated by addition of rec-hBD-3 at various concentrations (from 1 nM to 5 μM) for 48 h in FBS free medium. After the treatment, cells were triply washed with PBS, detached with trypsin, and counted in hemocytometer. The percentage of dead cells was analyzed using trypan blue staining.

To evaluate the effect of rec-hBD-3 on cell viability, MTT-test has been applied [15]. A549, A431, and TPC-1 cells were seeded into 96-well plates ($7x10^3$ cells per well) and incubated with rec-hBD-3 for 48 h in FBS free medium. Then the cells were treated with (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) by standard protocol, and colorimetric reaction was evaluated with the use of ELISA reader (Awareness Technology Inc, USA) at λ = 545.

The data are reported as the mean ± SD. The statistical significance of differences between mean values was assessed by the Student's *t*-test. Values $p < 0.05$ were considered statistically significant.

Results and discussion. For expression of rec-hBD-3 we have used GST-system for production of recombinant proteins allowing protein expression in *E.coli* strains in a form of chimeric proteins fused to GST tag. Both PCR product and pGEX-2T vector were purified, digested by BamHI and EcoRI restriction endonucleases, ligated and the hybrid plasmid was used for subsequent transformation of *E. coli BL21(DE3)* cells. After selection of colonies (Fig. 1), recombinant vectors were analyzed by digestion with BamHI and EcoRI endonucleases; the result evidenced on correct 135 bp insertion the sequence of which was shown to correspond to nucleotide sequence of hBD-3 gene by direct DNA sequencing analysis.

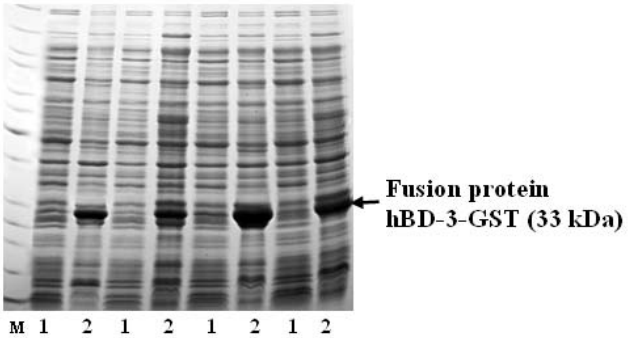

Fig. 1. SDS-PAGE analysis of lysates of bacterial colonies before (lines 1) and after (line 2) induction of bacteria with 1 mM IPTG for 6 h.; M – protein marker (SM0661, Fermentas)

Purification of rec-hBD-3 has been performed by routine three-step procedure including affine chromatography, proteolysis of fusion protein with thrombin and reverse phase chromatography. Purity of hBD-3 fraction was analyzed by gradient 7–22% SDS-PAGE (Fig. 2,a) and antimicrobial activity of the defensin was determined by inhibitory zone test. Rec-hBD-3 was active against *P. aeruginosa* at micromolar concentrations (Fig. 2, b). So, we have expressed in prokaryotic system and purified recombinant hBD-3 with antimicrobial activity.

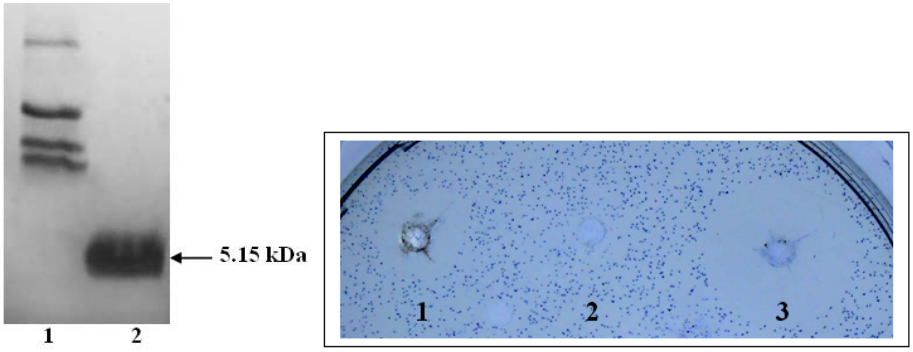

Fig. 2. A. SDS-PAGE analysis of rec-hBD-3 purified by reverse phase chromatography on Sep-Pak C18 (Water, USA).
1 – Protein Marker 6.5 – 200 kDa (SERVA Electrophoresis, Germany);
2 – fraction of rec-hBD-3 eluted with 50% CH3CN. B. Antimicrobial activity of rec-hBD-3 against *P.aeruginosa* in radial diffusion assay:
1 – 2 μg rec-hBD-3; 2 – 3 μL H_2O (control); 3 – 7 μg rec-hBD-3

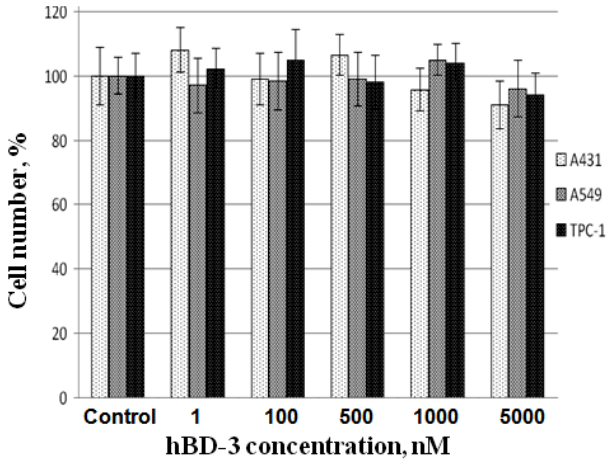

Fig. 3. Effect of rec-hBD-4 on proliferation of cultured cells of A431, A549, and TPC-1 lines.
The number of attached cells was evaluated by direct cell counting.
The data of three independent experiments are presented as the mean ± SD

Next, we have analyzed whether hBD-3 could affect cancer cell proliferation and viability in nanomolar concentrations. A concentration-dependent effects toward cancer cell growth were reported earlier for some beta-defensins, in particular hBD-2 and hBD-4 [5, 13]. The influence of rec-hBD-3 on proliferation and viability of three cultured human cancer cell lines – A431 (epidermoid carcinoma cells), A549 (lung adenocarcinoma cells), and TPC-1 (papillary thyroid cancer cells) was evaluated by direct cell counting technique and MTT assay respectively.

As it has been shown, rec-hBD-3 did not exert significant effects on proliferation and viability of cultured cancer cell lines at the range of concentrations from 1 nM to 5 μM (Fig. 3, 4). These data are in accordance with the data reported earlier on the absence of cytotoxic activity of this defensin at low micromolar concentrations [7]. Interestingly, when we have studied combined effect of rec-hBD-3 and rec-hBD-2 on viability of A431 cells (Fig. 5), we have registered significant increase of cytoxicity of such combination compared to that of rec-hBD-2 only.

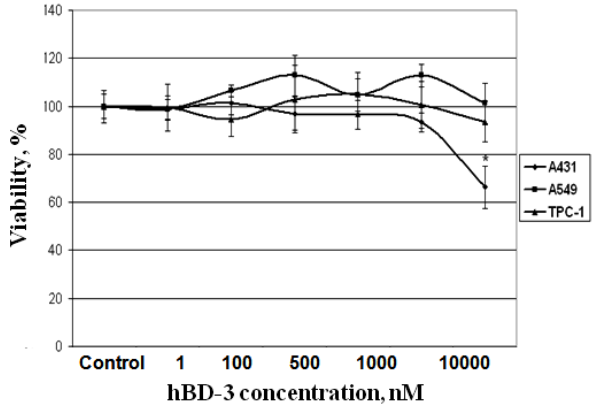

Fig. 4. Effect of rec-hBD-3 on viability of A431, A549, and TPC-1 cells.
The number of viable cells was evaluated by MTT analysis.
The data of three independent experiments are presented as the mean ± SD

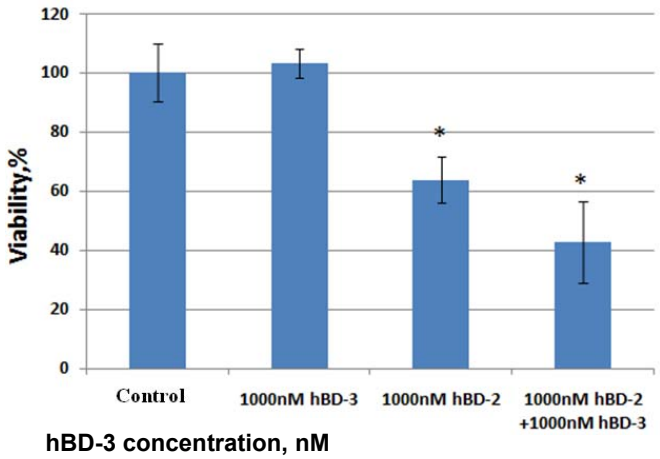

Fig. 5. Effect of rec-hBD-2 alone or in combination with rec-hBD-3 on viability of A431 cells.
The number of viable cells was evaluated by MTT analysis.
The data of three independent experiments are presented as the mean ± SD.
***The difference is significant compared to appropriate control value ($p < 0.05$)**

Moreover, while being noncytotoxic, rec-hBD-3 has been found to potentiate antiproliferative effects of clinically well-established anti-mitotic chemotherapy medication – docetaxel. As it has been demonstrated in MTT assay (Fig. 6), docetaxel at the doses of 0.1 and 1 μM significantly suppressed viability of A431 cells in a concentration dependent manner. The cytotoxic effect of 0.1 and 1 μM docetaxel in combination with 500 nM, 1 μM or 2 μM rec-hBD-3 was significantly higher than that of docetaxel only (Fig. 6). This finding could be of potential clinical importance, and the mechanisms of hBD-3-dependent attenuation of cytotoxic effects of docetaxel require further examination.

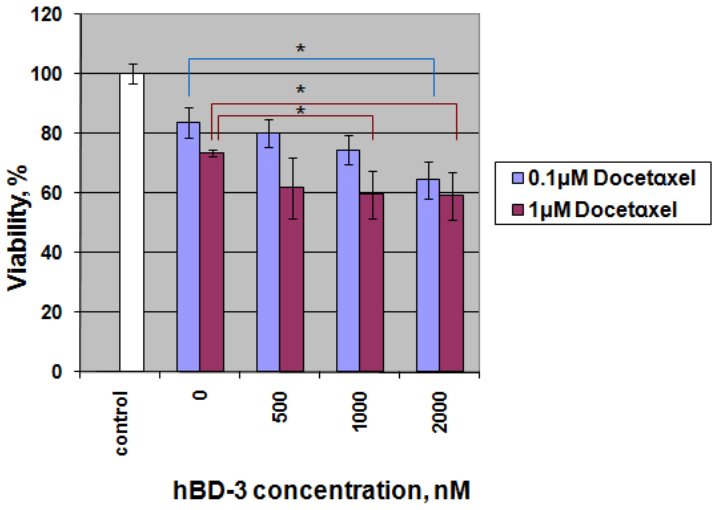

Fig. 6. Effect of docetaxel in combination with rec-hBD-3 on viability of A431 cells.
The number of viable cells was evaluated by MTT analysis. The data of three independent experiments are presented as the mean ± SD.
***The difference is significant compared to appropriate control value ($p < 0.05$)**

Concussion. In this work we report on procaryotic expression of bioactive rec-hBD-3. Analysis of biological activity of this defensin has shown that hBD-3 has no effect on proliferation and viability of cultured human cancer cells but it is capable to potentiate cytotoxic effects of hBD-2 and docetaxel *in vitro*.

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Abbreviations used: GST – glutathione-S-transferase; hBDs – human beta-defensins; hBD-3 – human beta-defensin-3; rec-hBD-3 – recombinant hBD-3.

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ЕКСПРЕСІЯ РЕКОМБІНАНТНОГО БЕТА-ДЕФЕНСИНУ-3 ЛЮДИНИ ТА АНАЛІЗ ЙОГО БІОЛОГІЧНОЇ АКТИВНОСТІ

Бета-дефенсини людини (human beta-defensins, hBDs) – це невеликі за розміром катіонні антимікробні пептиди з широким спектром біологічної активності. Метою дослідження було клонувати та експресувати в E.coli рекомбінантний бета-дефенсин-3 людини (rec-hBD-3), провести його очистку та проаналізувати in vitro біологічну активність цього пептиду. кДНК hBD-3 було клоновано у вектор pGEX-2T, надалі рекомбінантною плазмідою було трансформовано клітини E.coli BL21(DE3). Rec-hBD-3 було експресовано в бактеріальних клітинах у вигляді злитого білка GST-hBD-3 та очищено шляхом афінної хроматографії на глютатіон-агарозі, розщеплення злитого білка тромбіном та розділення продуктів протеолізу зворотньофазовою хроматографією на носії Sep-Pack C18. Аналіз біологічної активності rec-hBD-3 показав, що рекомбінантний пептид є активним проти Pseudomonas aeruginosa в мікромолярних концентраціях в тесті радіальної дифузії, не впливає на проліферацію та життєздатність культивованих пухлинних клітин людини ліній A431, A549 та TPC-1, але є здатним підсилювати цитотоксичні ефекти rec-hBD-2 та доцетакселу in vitro.

Ключові слова: бета-дефенсин-3 людини, пухлинна клітина, проліферація, життєздатність, антимікробна активність.

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ЭКСПРЕССИЯ РЕКОМБИНАНТНОГО БЕТА-ДЕФЕНСИНА-3 ЧЕЛОВЕКА И АНАЛИЗ ЕГО БИОЛОГИЧЕСКОЙ АКТИВНОСТИ

Бета-дефенсины человека (human beta-defensins. hBDs) – это катионные антимикробные пептиды с широким спектром биологической активности. Целью исследования было клонировать и экспрессировать в E.coli рекомбинантный бета-дефенсин-3 человека (rec-hBD-3), провести его очистку и проанализировать in vitro биологическую активность этого пептида. кДНК hBD-3 клонировали в вектор pGEX-2T, рекомбинантной плазмидой трансформировали клетки E.coli BL21(DE3). Rec-hBD-3 экспрессировали в бактериальных клетках в виде слитого белка GST-hBD-3 и очищали путем аффинной хроматографии на глютатион-агарозе, расщепления слитого белка тромбином и разделения продуктов протеолиза обратнофазовой хроматографией на Sep-Pack C18. Анализ биологической активности rec-hBD-3 показал, что рекомбинантный пептид активен против Pseudomonas aeruginosa в микромолярных концентрациях в тесте радиальной диффузии, не влияет на пролиферацию и жизнеспособность культивированных опухолевых клеток линий A431, A549 и TPC-1, однако способен усиливать цитотоксические эффекты rec-hBD-2 и доцетаксела in vitro.

Ключевые слова: бета-дефенсин-3 человека, опухолевая клетка, пролиферация, жизнеспособность, антимикробная активность.